PATENT
77670/495

APPLICATION UNDER UNITED STATES PATENT LAWS

Title of U.S. Patent No. 5,935,832: FARNESYL DIPHOSPHATE SYNTHASE

Inventors: Hiroyuki Nakane, Chikara Ohto, Shinichi Ohnuma, Kazutake Hirooka, Tokuzo Nishino

Attorneys: Kenyon & Kenyon
One Broadway
New York, New York 10004

Telephone: 212-425-7200

<u>This is a:</u>

[ ] Provisional Application

[ ] Regular Utility Application

[ ] Continuing Application

[ ] PCT National Phase Application

[ ] Design Application

[x] Reissue Application of U.S. Patent No. 5,935,832

[ ] Plant Application

SPECIFICATION

FARNESYL DIPHOSPHATE SYNTHASE

BACKGROUND OF INVENTION

1. Field of Invention

The present invention relates to a novel mutant enzyme which synthesizes linear prenyl diphosphates that are precursors of compounds, important for organisms, such as steroids, ubiquinones, dolichols, carotenoids, prenylated proteins, animal hormones, plant hormones, and the like; a genetic system encoding said enzyme; and a method for producing and using said enzyme.

2. Related Art

Of the substances having important functions in organisms, many are biosynthesized using isoprene (2-methyl-1,3-butadiene) as a constituent units. These compounds are also called isoprenoids, terpenoids, or terpenes, and are classified depending on the number of carbon atoms into hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes (C30), tetraterpenes (C40), and the like. The actual biosynthesis starts with the mevalonate pathway through which mevalonic acid-5-diphosphate is synthesized, followed by the synthesis of isopentenyl diphosphate (IPP) which is an active isoprene unit.

The identity of the isoprene unit that was proposed as a precursor was found to be isopentenyl diphosphate, the so-called active isoprene unit. Dimethylallyl diphosphate (DMAPP), an isomer of isopentenyl diphosphate, being used as a substrate in the synthesis of isopentenyl adenine which is known as a cytokinin, one of the plant hormones, it is also known to undergo a condensation reaction with isopentenyl diphosphate to synthesize chain-form active isoprenoids such as geranyl diphosphate (GPP), neryl diphosphate, farnesyl diphosphate (FPP), geranylgeranyl diphosphate (GGPP), geranylfarnesyl diphosphate (GFPP), hexaprenyl diphosphate (HexPP), heptaprenyl diphosphate (HepPP), and the like.

There are Z type and E type condensation reactions. Geranyl diphosphate is a product of E type condensation and neryl diphosphate is of Z type condensation. Although, the all-E type is considered to be the active form in farnesyl diphosphate and geranylgeranyl diphosphate, the Z type condensation reaction leads to the synthesis of natural rubber, dolichols, bactoprenols (undecaprenols), and plants various polyprenols found in. They are believed to undergo the condensation reaction using the phosphate ester bond energy of the pyrophosphate and the carbon backbone present in the molecule and to produce pyrophosphate as the byproduct of the reaction.

Farnesyl diphosphate or geranylgeranyl diphosphate serve as a reaction substrate leading to the synthesis of prenylated proteins (from farnesyl diphosphate or geranylgeranyl diphosphate) represented by G proteins that are important in the mechanism of signal transducer in the cell; cell membrane lipids (from geranylgeranyl diphosphate) of archaea; squalene (from farnesyl diphosphate) which is a precursor of steroids; and phytoene (from geranylgeranyl diphosphate) which is a precursor of carotenoids. Prenyl diphosphates from hexaprenyl diphosphate and heptaprenyl diphosphate having six and seven isoprene units, respectively, to prenyl diphosphates having ten isoprene units serve as the precursors of the synthesis of ubiquinone and menaquinone (vitamin K2) that work in the electron transport system.

Furthermore, via the biosynthesis of these active-form isoprenoids, a vast number of kinds of compounds that are

vital to life have been synthesized. Just to mention a few, there are cytokinins that are plant hormones and isopentenyl adenosine-modified tRNA that use hemiterpenes as their precursor of synthesis, geraniols and that isomer nerol belonging to monoterpens are the main components of rose oil perfume and a camphor tree extract, camphor, which is an insecticide. Sesquihormones include juvenile hormones of insects, diterpenes include a plant hormone gibberellin, trail pheromones of insects, and retinols and retinals that function as the visual pigment precursors, binding components of the purple membrane proteins of highly halophilic archaea, and vitamin A.

Furthermore, using squalene, a triterpene, a wide variety of steroid compounds have been synthesized, including, for example, animal sex hormones, vitamin D, ecdysone which is an ecdysis hormone of insects, a plant hormone brassinolide, constitution of the plasma membrane etc. Various carotenoids of tetraterpenes that are precursors of various pigments of organisms and vitamin A are also important compounds derived from active isoprenoids. Compounds such as chlorophyll, pheophytin, tocopherol (vitamin E), and phylloquinone (vitamin K1) are also derived from tetraterpenes.

The active isoprenoid synthases that sequentially condense isopentenyl diphosphates with such allylic substrates as dimethylallyl diphosphate, geranyl diphosphate, farnesyl diphosphate, geranylgeranyl diphosphate, geranylfarnesyl diphosphate, etc. are called the prenyl diphosphate synthases, and are also called, based on the name of the compound having the maximum chain length of the major reaction products, for example farnesyl diphosphate synthase (FPP synthase), geranylgeranyl diphosphate (GGPP synthase), and the like. There are reports on purification, activity measurement, genetic cloning, and sequencing of the DNA encoding enzymes such as farnesyl diphosphate synthase, geranylgeranyl diphosphate synthase, hexaprenyl diphosphate synthase, heptaprenyl diphosphate synthase, octaprenyl diphosphate synthase, nonaprenyl diphosphate synthase (solanesyl diphosphate synthase), undecaprenyl diphosphate synthase, and the like from bacteria, archaea, fungi, plants, and animals.

These active isoprenoid synthases constituting the basis of chemical synthesis of a great variety of compounds that are important both in the industry and in the academic field of life sciences have had few practical uses in the industrial application due to their unstable nature and low specific activities. However, with the isolation of thermostable prenyl diphosphate synthases from thermophilic bacteria and archaea and the genes encoding these enzymes, their availability as the enzyme has increased.

With regard to farnesyl diphosphate synthase, a gene was isolated from *Bacillus stearothermophilus,* a medium thermophile, and an enzyme having a medium thermal stability was prepared using *Escherichia coli* as host cell [T. Koyama et al. (1993) J. Biochem., 113: 355–363; Japanese Unexamined Patent Publication No. 5(1993)-219961]. With regard to geranylgeranyl diphosphate synthase, a gene was isolated from high thermophiles such as *Sulfolobus acidocaldarius* and *Thermus thermophilus* [S. -i. Ohnuma et al., (1994) J. Biol. Chem., 269: 14792–14797; Japanese Unexamined Patent Publication No. 7(1995)-308193, and; Japanese Unexamined Patent Publication No. 7(1995)-294956], and enzymes having a high thermal stability were prepared.

Furthermore, with regard to the prenyl diphosphate synthase having the functions of both of the farnesyl diphosphate synthase and the geranylgeranyl diphosphate synthase,

from *Rhodobactor sphaeroides*, RCPHSYNG from *Rhodobactor capsulatus*, EHCRTS.pe from *Erwinia herbicola*, MXCRTNODA from *Myxococcus thaliana*, and NCAL3.pep from *Neurospora crassa*. The number indicated on the left of each amino acid sequence represents the site from the N-terminal side of each geranylgeranyl diphosphate synthase at the N-terminal of the amino acid sequence.

FIG. 2 is a graph showing the thermal stability of the mutant prenyl diphosphate synthase. The ordinate shows the relative activity to 100% at incubation at 60° C. The abscissa shows the incubation temperature. SacGGPS is the geranylgeranyl diphosphate synthase prior to mutation. The others represent the mutant type enzyme of each. BstFPS is the farnesyl diphosphate synthase derived from *Bacillus stearothermophillus*.

FIG. 3 shows a photograph of a development pattern of thin layer chromatography of the dephosphorylated reaction products of the mutant prenyl diphosphate synthase when geranyl diphosphate was used as the allylic substrate. In the figure, ori. represents the origin of development, and s.f. represents the solvent front.

GOH is geraniol, FOH is farnesol, GGOH is geranyl geraniol, and GFOH is geranylfarnesol, and these are produced from dephosphorylation of geranyl diphosphate, farnesyl phosphate, geranylgeranyl diphosphate, and geranylfarnesyl diphosphate, respectively. SacGGPS is the geranylgeranyl diphosphate synthase prior to mutation. The others are each mutant enzymes.

DETAILED DESCRIPTION

It has been proposed that there are five conserved regions in the amino acid sequence of a prenyl diphosphate synthase (one subunit in the case of a heterodimer) [A. Chem et al., Protein Science Vol. 3, pp. 600–607, 1994]. It is also known that of the five conserved regions, there is an aspartic acid-rich domain conserved sequence I [DDXX(XX)D] (wherein X denotes any amino acid, and the two X's in the parentheses may not be present) in region II. Although there is also an aspartic acid-rich domain indicated as "DDXXD" in region V, the aspartic acid-rich domain used to specify the modified region of the amino acid sequence of the present invention is the one present in region II, and this domain is termed as the aspartic acid-rich domain I as compared to the aspartic acid-rich domain II present in region V.

As the prenyl diphosphate synthases having the aspartic acid-rich domain as described above, there can be mentioned farnesyl diphosphate synthase, geranylgeranyl diphosphate synthase, hexaprenyl diphosphate synthase, heptaprenyl diphosphate synthase, octaprenyl diphosphate synthase, nonaprenyl diphosphate synthase, undecaprenyl diphosphate synthase, and the like. More specific examples include the farnesyl diphosphate synthase of *Bacillus stearothermophilus*, the farnesyl diphosphate synthase of *Escherichia coli*, the farnesyl diphosphate synthase of *Saccharomyces cerevisiae*, the farnesyl diphosphate synthase of the rat, the farnesyl diphosphate synthase of the human, the geranylgeranyl diphosphate synthase of *Neurospora crassa*, the hexaprenyl diphosphate synthase of *Saccharomyces cerevisiae*, and the like.

By way of example of some of these, regions I to V and the aspartic acid-rich domain I (in the box) in region II of the amino acid sequence of geranylgeranyl diphosphate synthases are shown in FIG. 1.

The present invention can be applied to any prenyl diphosphate synthase having the aspartic acid-rich domain I.

In accordance with the present invention, in the amino acid sequence of a prenyl diphosphate synthase, at least one amino acid residue selected from (a) the amino acid residues in between the amino acid residue located at the fifth position in the N-terminal direction from D of the N-terminal and the amino acid residue located at the first position in the N-terminal direction from D of said N-terminal of the aspartic acid-rich domain DDXX(XX)D (wherein X denotes any amino acid, and the two X's in the parentheses may not be present) present in region II, and (b) the amino acid residue located at the first position in the N-terminal direction from D of the C-terminal of said aspartic acid-rich domain has been substituted by another amino acid, and/or

an additional one or more amino acids have been inserted in between the amino acid residue located at the first position in the N-terminal side from D of the C-terminal and D of said C-terminal of said aspartic acid-rich domain.

The mutant prenyl diphosphate synthase of the present invention can synthesize a farnesyl diphosphate having a shorter chain length than the prenyl diphosphate synthesized by the native prenyl diphosphate synthase.

In accordance with the present invention, by way of example, the gene of the geranylgeranyl diphosphate synthase of a highly thermophilic archaea, *Sulfolobus acidocaldarius*, is used as the starting material. *Sulfolobus acidocaldarius* is available from ATCC as ATCC No. 33909. The method for cloning the gene has been described in detail in Japanese Unexamined Patent Publication No. 7-308193. It has also been disclosed with the accession No. D28748 in the gene information data base such as GenBank. By using the sequence it can be cloned in the conventional method known in the art. An example of the other cloning methods is illustrated in Example 1 herein and its nucleotide sequence is shown as SEQ ID No: 2.

More specifically, the mutant enzyme of the present invention is a mutant prenyl diphosphate synthase characterized in that at least one amino acid selected from phenylalanine in position 77, threonine at position 78, valine at position 80, histidine at position 81, and isoleucine at position 84 has been substituted by another amino acid, and/or amino acid(s) have been inserted in between isoleucine at position 84 and methionine at position 85 in the geranylgeranyl diphosphate synthase having the amino acid sequence as set forth in SEQ ID No: 1.

By way of example, there is provided the amino acid sequences wherein the amino acids have been substituted as shown below:

Mutant enzyme 1: Changes from threonine at position 78 to phenylalanine, and histidine at position 81 to alanine;

Mutant enzyme 2: Changes from threonine at position 78 to phenylalanine, and histidine at position 81 to leucine;

Mutant enzyme 3: Changes from phenylalanine at position 77 to tyrosine, threonine at position 78 to phenylalanine, and histidine at position 81 to leucine;

Mutant enzyme 4: Changes from phenylalanine at position 77 to tyrosine, threonine at position 78 to phenylalanine, and histidine at position 81 to alanine;

Mutant enzyme 5: Changes from phenylalanine at position 77 to tyrosine, threonine at position 78 to serine, valine at position 80 to isoleucine, and isoleucine at position 84 to leucine, and an insertion of proline and serine in between isoleucine at position 84 and methionine at position 85.

In accordance with the present invention, it is indicated that the mutant prenyl diphosphate synthase retains the characteristic properties that were owned by the native prenyl diphosphate synthase. By way of example, the above-

As the enzyme, not only a purified enzyme but also a crude enzyme that may be semi-purified to various stages, or a mixture of the cultured broth of a microorganism may be used. Alternatively there may be used immobilized enzymes prepared according to the general method from said enzyme, said crude enzyme, or a product containing the enzyme.

As the substrate, there may be used dimethyl allyl diphosphates or geranyl diphosphates and isopentenyl diphosphates. As the reaction medium, water or an aqueous buffer solution, for example Tris buffer or phosphate buffer and the like, may be used.

By using the method of producing the mutant prenyl diphosphate synthase obtained by the present invention, the mutant prenyl diphosphate synthase derived from a archaea may be created that is more stable and thus easier to handle and that produces prrenyl diphosphate. Furthermore, there is also expected a creation of the farnesyl diphosphate-producing mutant prenyl diphosphate synthase that has the property of the prenyl diphosphate synthase prior to mutation (for example, salt stability or stability in a wide range of pH) added thereto.

In the claims and the specification of the present invention, amino acid residues are expressed by the one-letter codes or three-letter codes as described hereinbelow:

A; Ala; alanine
C; Cys; cysteine
D; Asp; aspartic acid
E; Glu; glutamic acid
F; Phe; phenylalanine
G; Gly; glycine
H; His; histidine
I; Ile; isoleucine
K: Lys; lysine
L; Leu; leucine
M; Met; methionine
N; Asn; asparagine
P; Pro; proline
Q; Gln; glutamine
R; Arg; arginine
S; Ser; serine
T; Thr; threonine
V; Val; valine
W; Trp; tryptophan
Y; Tyr; tyrosine

Substitution of amino acid is expressed in the order of "the amino acid residue before substitution," "number of the amino acid residue," and "the amino acid residue after substitution," by the one-letter codes of amino acids. For example, the mutation in which a tyrosine residue at position 81 is replaced with a methionine residue is expressed as Y81M. Furthermore, the insertion of amino acid residues is expressed by "the number of the amino acid residue at the N-terminal side of the insertion site prior to insertion," "the amino acid residue that was inserted," and "the number of the amino acid residue at the C-terminal side of the insertion site prior to insertion." For example, the insertion of alanine in between the amino acid at position 84 and the amino acid at position 85 is expressed as 84A85.

EXAMPLES

The present invention is now explained with reference to specific examples, but they must not be construed to limit the invention in any way.

Example 1

Construction of a Plasmid Containing the Gene for Geranylaeranyl Diphosphate Synthase

The gene for the geranylgeranyl diphosphate synthase (hereinafter referred to as SacGGPS) derived from *Sulfolobus acidocaldarius* was subcloned at the HindIII site of the plasmid vector pBluescript II (KS+) commercially available from Toyoboseki. The plasmid DNA was designated as pBs-SacGGPS. The SacGGPS gene is available from *Escherichia coli* DH5α (pGGPS1) that was internationally deposited on Jan. 31, 1994 with the National Institute of Bioscience and Human Technology Agency of Industrial Science and Technology, of Ibalaki, Japan under the accession number of FERM BP-4982.

Also, the entire nucleotide sequence of the SacGGPS gene has been published in Japanese Unexamined Patent Publication No. 7-308193 Shin-ichi Ohnuma et al. (1994) The Journal of Biological Chemistry Vol. 269:14792–14797, or in the genetic information data bank such as GenBank under the accession number D28748. Since *Sulfolobus acidocaldarius* is also available from various depositories of microorganisms such as ATCC etc. (as ATCC No. 33909), the DNA of the gene region of SacGGPS can be obtained by the conventional gene cloning method.

Example 2

Synthesis of the Oligonucleotides for Introducing Mutation

For introducing mutation of the gene of geranylgeranyl diphosphate synthase, the following oligonucleotides were designed and synthesized:

Primer DNA (T78F, H81A):
5'-CATACTTTTTTCCTTGTGGCTGATGATATCATGGATC-3' (SEQ ID No: 3)
Primer DNA (T78F, H81L):
5'-CATACTTTTTTCCTTGTGCTTGATGATATCATGGATC-3' (SEQ ID No: 4)
Primer DNA (F77Y, T78F, H81L):
5'-CATACTTATTTCCTTGTGCTTGATGATATCATGGATC-3' (SEQ ID No: 5)
Primer DNA (F77Y, T78F, H81A):
5'-CATACTTATTTCCTTGTGGCTGATGATATCATGGATC-3' (SEQ ID No: 6)
Primer DNA (F77Y, T78S, V80I, I84L, 84PS85):
5'-GTTCTTCATACTTATTCGCTTATTCATGATAGTATT-3' (SEQ ID No: 7), and 5'-ATTCATGATGATCTTCCATCGATGGATCAAGAT-3' (SEQ ID No: 8).

Introduction of the mutation (F77Y, T78S, V80I, I84L, 84PS85) was effected using two nucleotides. First, mutation was introduced as mentioned in Example 3 using the oligonucleotide

5'-GTTCTTCATACTTATTCGCTTATTCATGATAGTATT-31 (SEQ ID No: 7) and a transformant was prepared in accordance with Example 4, and furthermore mutation was introduced into the plasmid thus obtained using the oligonucleotide

5'-ATTCATGATGATCTTCCATCGATGGATCAAGAT-3' (SEQ ID No: 8).

These nucleotides have a mutation in the codon encoding at least one amino acid residue selected from phenylalanine at position 77, threonine at position 78, valine at position 80, histidine at position 81, and isoleucine at *position 84* in SacGGPS. In addition to the introduction of the codon